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Noise induces up-regulation of $P2X_2$ receptor subunit of ATP-gated ion channels in the rat cochlea

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Regulation of $P2X_2$ receptor ($P2X_2R$) expression in the rat cochlea in response to noise was analysed. Sustained loud sound (90–120 dB white noise, > 6 h), increased $P2X_2R$ mRNA and protein levels in rat organ of Corti and spiral ganglion (primary auditory neurones). $P2X_2R$ expression by the type I spiral ganglion neurones, which innervate the inner hair cells via the inner spiral plexus, was confirmed by confocal immunofluorescence. This also revealed increased $P2X_2R$ labelling of outer hair cell (OHC) stereocilia and cuticular plates, reflecting trafficking of greater numbers of ATP-gated ion channels assembled with $P2X_2R$ subunits to the transducer site. Whole-cell voltage clamp of OHC confirmed the noise-induced up-regulation of ATP-gated inward currents. These data indicate that regulation of $P2X_2$ receptor gene expression in the cochlea is adaptive, with sustained loud sound promoting increased transcription and translation specifically at sites regulating hearing sensitivity and auditory neurotransmission. *NeuroReport* 14:817–823 © 2003 Lippincott Williams & Wilkins.

Key words: Auditory neurotransmission; Cochlea; Gene regulation; Hearing; Outer hair cells; Purinoceptor; Spiral ganglion; Sound transduction

INTRODUCTION

Purinoceptor signalling mediates the actions of extracellular ATP in cochlear physiology, with a body of data from *in vitro* and *in vivo* experiments indicating roles in sound transduction, associated cochlear electrochemical homeostasis, and auditory neurotransmission [1]. It also appears likely that stressor-induced release of ATP in cochlear tissues contributes to cochlear pathologies and hence hearing loss [2]. Among the purinoceptor family, $P2X_2$ receptors ($P2X_2R$), which assemble as subunits of ATP-gated ion channels, are expressed at high levels by the cochlear hair cells, spiral ganglion neurones, including synaptic terminals with the inner and outer hair cells [3–7], and by cells lining the cochlear partition [4,8,9].

Release of ATP into cochlear fluids occurs during loud sound [10] and elevations of ATP in the endolymph cause a P2X receptor (P2XR)-mediated reduction in endocochlear potential, and hearing loss [11,12]. $P2X_2R$ localised to the hair cell stereocilia (the site of sound transduction) and cuticular plate region (facing the endolymphatic compartment), complement the global ATP-activated shunt from P2XR-mediated conductances of other epithelial cell types lining the scala media, limiting sound transduction [4,13,14]. This shunt pathway is linked with the para-epithelial movement of K^+ that resupplies the vascular stria, particularly through the outer sulcus cell region, which may be modulated by ATP-dependent activation of $P2X_2R$ [15].

P2XR may also serve as modulators of glutamatergic auditory neurotransmission. $P2X_2R$ have been localised to post-synaptic terminals of the inner hair cell (IHC)–type I spiral ganglion neurone (sgnI), and outer hair cell (OHC)–type II spiral ganglion neurone (sgnII) synapses [4]. Given that glutamate is the primary neurotransmitter, acting via AMPA-type receptors at the inner hair cell–sng I synapse [16], ATP may be co-released with glutamate by the hair cells to modulate auditory coding. The recent identification of $P2X_7$ receptor expression by putative efferent terminals on the OHC suggests that ATP may also act pre-synaptically to modify cholinergic efferent inhibition of the OHC [17].

$P2X_2R$ are under dynamic transcriptional and post-translational control. For example, ATP-gated ion channel expression changes within a few hours when spiral ganglion neurones are isolated [7]. Expression studies using P2XR-GFP reporter gene chimeras indicate that the receptors are in dynamic flux within cells, and respond to agonist binding by changes in receptor clustering and receptor turnover [18,19]. Given that the agonist for P2XR is extracellular ATP,

and that ATP release into cochlear fluids is facilitated by noise exposure, we investigated the potential for regulation of $P2X_2R$ transcription, translation and protein targeting within cochlear tissues in response to loud sound.

MATERIALS AND METHODS

The care and use of the animals as reported here was approved by the University of Auckland Animal Ethics Committee and the Committee for Animal Subjects of the San Diego VA Medical Center.

Detection of $P2X_2R$ mRNA: For *in situ* hybridisation labelling of $P2X_2R$ mRNA, young adult (200–300 g) rats (Sprague–Dawley strain) were exposed to white noise (1414–5656 Hz; 100–120 dB SPL, 1–24 h) in a sound chamber. Control rats were held in the laboratory at ambient sound levels. Following noise exposure, the test and control rats were anaesthetised with pentobarbital and perfused transcardially with acid shift paraformaldehyde in 0.1 M phosphate buffer (PB). Dissected cochleas were post-fixed then decalcified, cryosectioned (20 μm) onto slides (Superfrost Plus, Fisher Scientific), which were stored at −70°C. The *in situ* hybridisation protocol utilised [^{35}S]-labelled cRNA probes derived from $P2X_2R$ cDNA cloned into the pCRII vector as described previously [9,20]. Sections from both control and noise-exposed cochleas were batch processed together. The sections were permeabilised with 0.001% proteinase K prior to hybridisation at 60°C overnight in buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 0.5% tRNA, 10 mM dithiothreitol, 1 × Denhardt's solution and 10% dextran sulphate. After hybridisation, sections were treated with ribonuclease A, and washed in 0.1 × saline sodium citrate (SSC) and 1 mM DTT at 65°C, then dehydrated, coated with autoradiographic emulsion (NTB-2, Kodak) and exposed at 4°C for 10–14 days. Slides were developed (D-19, Kodak), fixed, then counterstained with the fluorescent dye bis-benzimide.

$P2X_2R$ subunit immunolabelling: Immunoperoxidase experiments followed the procedure used to show our original description of $P2X_2$ immunolabelling in the rat cochlea [8]. Rats (Wistar strain) were contained either at ambient sound levels of the laboratory, or placed within a sound chamber and exposed to 90 dB SPL of white noise (1–17 kHz) for 3 days. At the conclusion of the noise exposure the animals were anaesthetised with pentobarbital and perfused with 4% formaldehyde and 0.5% glutaraldehyde, in PB (pH 7.4). Cryosections (20 μm) of decapsulated adult rat cochlea, either control or noise-exposed, were mounted on slides (ProbeOn Plus, Fisher Scientific), air-dried, and stored at −20°C. For qualitative comparison of $P2X_2R$ immunolabelling, cochleas from seven control and seven noise-exposed rats were processed in parallel. The sections were rehydrated in 0.1 M PBS and then blocked with 1.5% normal goat serum (NGS, Vector Laboratories, USA) for 2–3 h prior to overnight incubation (4°C) with the primary antibody ($P2X_2R96ab$; [20]) at 1:2000 dilution (including 1.5% NGS). The sections were washed and then incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, 1:200 dilution in 1.5% NGS) for 40 min at room temperature. The tissues were again washed (including an overnight soak) and antibody binding was detected using ABC reagent, and diaminobenzidine (DAB)-peroxidase (Vectastain ABC kit, Vector Laboratories). Parasagittal cryosections of rat cerebellum provided a positive control; negative controls omitted primary antibody. Peptide block controls have already been achieved with this antibody in guinea-pig, rat and mouse cochlear and brain tissues [8,14,20].

Confocal immunofluorescence provided quantitative analysis of changes of $P2X_2R$ expression in the cochlea induced by noise exposure. The study used cochleas from four control rats and four rats exposed to white noise (90 dB SPL, 72 h). The experiments used 50 μm floating cochlear sections permeabilised with 1% Triton X-100 and blocked with 10% NGS (40 min), prior to application of the $P2X_2R96ab$ antibody (1:1000) in 5% NGS, 0.1% Triton X-100 overnight (4°C, shaking). Following washes, CY3-labelled goat anti-rabbit secondary antibody (1:500, Chemicon) was applied for 2 h at room temperature, continuing for 2 h at 4°C. The tissue was then re-washed, including an overnight soak at 4°C, and then mounted on slides using Citifluor medium (Agar Scientific, UK). Immunofluorescence was observed using a confocal microscope, (TCS DB4, Leica GmbH; excitation 458/476 nm, emission 490–600 nm). Both spiral ganglion and organ of Corti were imaged for control and noise-exposed tissue alternately, using identical settings. For each field, five optical sections were obtained at 2 μm spacing, commencing within 5 μm of the surface. Image analysis was performed on an optical section from the centre of the stack. Image-Pro Plus (ver. 4.5.1, Media Cybernetics) software was used to demarcate cell-type boundaries and determine mean pixel intensities above background.

Recording of outer hair cell ATP-gated currents: Whole-cell voltage clamp studies were performed to verify functional ATP-gated ion channel (P2X receptor) responses by OHC from control and noise-exposed (90 dB SPL, 72 h) rats. The procedure for OHC isolation followed that reported previously [4]. Dissected organ of Corti was treated with 0.25 mg/ml trypsin (Calbiochem) for 2 min, triturated and dispensed into a recording chamber on an inverted microscope (Nikon TMD, Japan). Whole-cell recordings with a mean series resistance of $8.7 \pm 0.6\,M\Omega$ ($n = 31$) were made from OHC from noise-exposed, and control rat cochleas (external solution in mM: NaCl 140, KCl 4.0, $CaCl_2$ 1.5, $MgCl_2$ 10.0, Na_2HPO_4 8.0, NaH_2PO_4 2.0, D-glucose 1.0, adjusted to pH 7.25 with NaOH, osmolarity 320 mOsm/l; internal solution: KCl 150, $MgCl_2$ 2.0, $CaCl_2$ 0.01, Na_2HPO_4 8.0, NaH_2PO_4 1.0, ethylene glycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) 0.5, D-glucose 3.0, adjusted to pH 7.25 with KOH; osmolarity 310 mOsm/l). OHC were voltage clamped (95% series resistance compensation) at a holding potential of −60 mV, and ATP (Sigma) was pressure-applied through a glass micropipette. Voltage clamp used an Axoclamp 200 patch clamp amplifier interfaced to a Digidata1200 controller, with pClamp 8 software (Axon Instruments, USA).

Data are presented as mean ± s.e.m. Statistical analysis utilised unpaired Student's *t*-tests to compare $P2X_2R$ immunolabelling and amplitude of OHC ATP-gated currents, in control and noise-treatment groups.

RESULTS

Effect of noise exposure on $P2X_2R$ mRNA expression: *In situ* hybridisation (ISH) localised $P2X_2R$ mRNA transcripts principally within the sensory epithelium of the cochlear partition, organ of Corti and spiral ganglion, as previously described [9]. In control sections, labelling was pronounced in the cochlear partition from the interdental cells at the insertion point of Reissner's membrane, through the epithelial cells of the inner sulcus, the hair cell region, to the outer sulcus up to the basal edge (spiral prominence) of the vascular stria (Fig. 1a). Labelling in the region of the outer hair cells was weaker than adjacent areas. No signal was evident in the vascular stria. Reissner's membrane was weakly labelled. The spiral ganglion region showed moderate labelling in foci consistent with $P2X_2R$ mRNA within the soma of individual spiral ganglion (SG) neurones.

Noise exposure induced an up-regulation of $P2X_2R$ mRNA expression in the cochlea. This was not evident in animals exposed to loud noise (100–120 dB) for 1–4 h ($n = 13$), but became apparent after 6 h noise exposure (not shown, $n = 4$) when there were moderate increases of signal in the organ of Corti, and a slight increase in the SG. However, after 24 h of noise treatment ($n = 18$) there were strong increases in $P2X_2R$ mRNA signal in the organ of Corti, particularly in the region of the OHC and Deiters' cells (DC), and moderate to strong increases in SG (Fig. 1b). The weak labelling in Reissner's membrane was unchanged and the vascular stria remained unlabelled. The $P2X_2R$ expression in the vestibular system was also unaffected by the noise treatment, with the levels of $P2X_2R$ mRNA labelling in the transitional cells of the crista ampullaris unchanged after noise exposure (not shown).

Effect of noise exposure on $P2X_2R$ immunolabelling: Immunohistochemistry was used to assess the effect of 72 h of 90 dB SPL white noise on $P2X_2R$ protein expression. Preliminary experiments using the immunoperoxidase technique confirmed the localisation of $P2X_2R$ expression indicated by $P2X_2R$ cRNA hybridisation, and was comparable to that provided in our previous immunoperoxidase localisation of $P2X_2R$ protein in the adult rat cochlea [8]. Within the organ of Corti, this included staining of the DC and the IHC regions, as well as the apical region and footplates of the pillar cells. The OHC, and the mid-region of the pillar cells were generally unlabelled. Immunolabelling was absent from the vascular stria, but present in the outer sulcus and spiral ligament, inner sulcus and spiral limbus. Staining in the tectorial membrane was attributed to

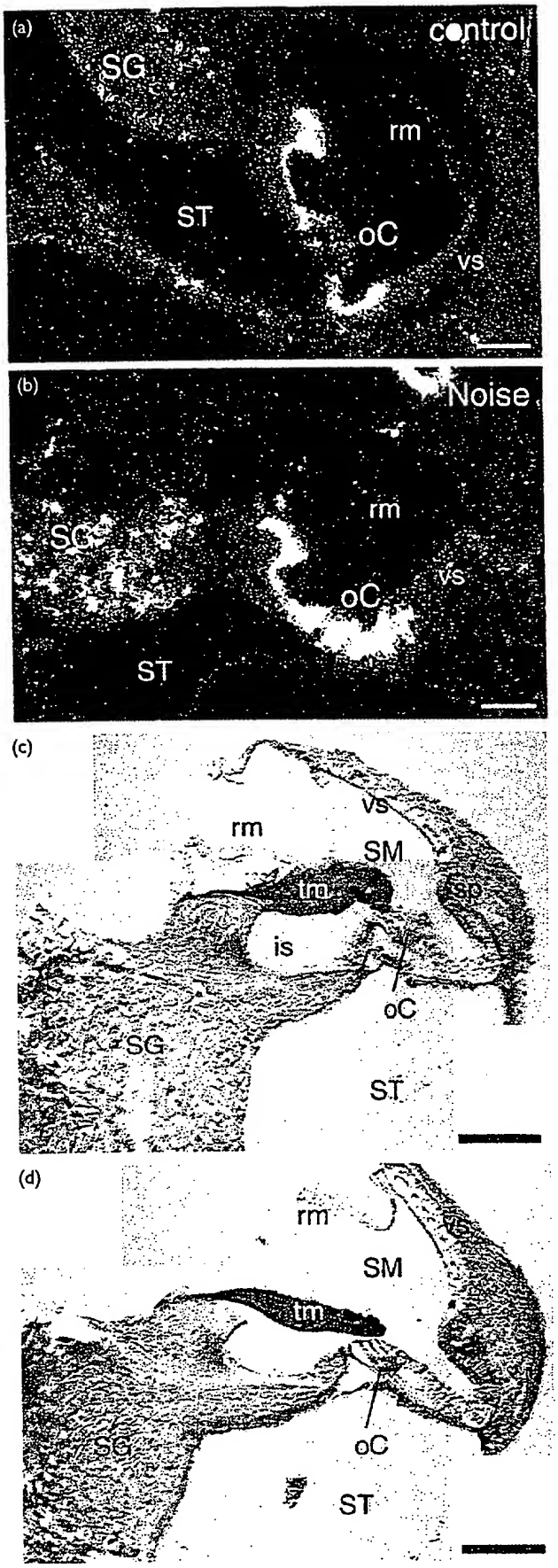

Fig. 1. Up-regulation of $P2X_2R$ mRNA and protein levels in rat cochlear spiral ganglion and organ of Corti following noise exposure (120 dB SPL, 24 h). (a) Control *in situ* hybridisation localising $P2X_2R$ mRNA in the organ of Corti, and inner and outer sulcus regions. Spiral ganglion (SG) neurones also show labelling. (b) Noise exposure (24 h) increased expression of $P2X_2R$ mRNA transcript levels in the SG and in the hair cell/Deiters' cell region of the organ of Corti. (c) Immunoperoxidase labelling of control rat cochlea for the P2X2R shows moderate signal in the SG and weaker signal in the organ of Corti region. (d) Section from noise-exposed cochlea (90 dB SPL, 72 h). Note the increase in $P2X_2R$ immunopositive signal within the SG and the organ of Corti. is, inner sulcus; oC, organ of Corti; rm, Reissner's membrane; SM scala media; sp, spiral prominence; ST, scala tympani; tm, tectorial membrane; vs, vascular stria. Bars = 100 µm.

non-specific binding as previously reported [8]. The SG showed variable labelling of individual neurones. Sections were unstained when the primary antibody was omitted (not shown). The SG and organ of Corti responded to the noise stimulus with increased $P2X_2R$ protein translation (Fig. 1c,d). In SG, not only did the apparent staining intensity increase, but the proportion of neurones demonstrating moderate staining also increased. In contrast, areas such as Reissner's membrane exhibited unaltered low-level staining. The vascular stria remained devoid of $P2X_2R$ immunolabelling.

Confocal immunofluorescence, in conjunction with semi-quantitative image analysis, was used to resolve the effect of noise exposure on $P2X_2R$ expression at the cellular level within the organ of Corti and SG (Fig. 2a–g). The organ of Corti showed a similar pattern of immunofluorescence labelling to that seen with the immunoperoxidase experiments. There was minimal staining of the OHC and the medial regions of the pillar cells (Fig. 2a,c), whereas the IHC region and DC exhibited a stronger signal. The tectorial membrane showed non-specific absorption of the primary antiserum. Sections lacked significant fluorescence when the primary antibody was omitted (Fig. 2e). Labelling occurred in most SG neurones, with considerable cytoplasmic signal (Fig. 2f).

Noise exposure caused an increase in the number of sections displaying bright labelling of OHC cuticular plates (Fig. 2b,d), and associated labelling of the apical half of the stereocilia of the OHC became more apparent (Fig. 2b,d). DC and IHC continued to exhibit considerable signal. Immunofluorescence within the SG increased markedly following noise exposure (Fig. 2f,g). To quantify the extent of the immunofluorescence signal, the mean pixel intensities above background for regions of the organ of Corti were determined within manually prescribed cell boundaries (Fig. 2h). The OHC cell body, from the nucleus to the cuticular plates, exhibited the lowest signal, and this was not affected by noise exposure (mean pixel intensity 38.6 ± 2.9, $n = 45$, control; 35.0 ± 1.7, $n = 58$, noise; $p > 0.05$). The stable low-level signal from the supranuclear region of the OHC was used as a reference for examining the proportional change in signal in the other areas of interest (Fig. 2i). The DC region had a mean pixel intensity 48% above the OHC reference in the control sections, which was unchanged by noise exposure ($p > 0.05$). The IHC region was 65% brighter in control tissue than the OHC reference, increasing to 110% following noise exposure ($p < 0.05$). This noise-induced increase in the $P2X_2R$ immunofluorescence signal in the IHC region may reflect the trafficking of additional $P2X_2R$ by spiral ganglion neurones to their distal neurite synaptic complex with the IHC, as well as increased $P2X_2R$ expression by associated supporting cells (border cells, inner phalangeal cells and inner pillar cells). The OHC cuticular plate region showed the largest sound-induced immunofluorescence increase, from 26% above the supranuclear OHC reference in controls, to 92%. This probably reflects the specific targeting of OHC $P2X_2R$ protein translation, and was evident in all cochlear regions examined (mid to apex). The SG fluorescence signal increased by 27% with noise exposure, from a mean pixel intensity of 86.5 ± 4.5 ($n = 38$) to 109.5 ± 22.7 ($n = 53$; Fig. 2h). In contrast, the cochlear nerve medial to the ganglion, which provided an internal reference in these fields, was unaffected (55.3 ± 3.4, control; 56.8 ± 2.1 noise). In the control tissue, the relative pixel intensity of the SG was 63% above the adjacent reference nerve fibre region. This increased to twice the reference level in noise-exposed tissue (Fig. 2i; $p < 0.001$).

Effect of noise exposure on outer hair cell ATP-gated inward currents: OHC isolated from all regions of the rat organ of Corti were selected on the basis of intact stereocilia, membrane birefringence and lack of Brownian motion in the cytoplasm. These cells had average cell lengths of $32.6 \pm 1.5\,\mu m$ and $27.4 \pm 2.0\,\mu m$, and widths of $9.3 \pm 0.2\,\mu m$ and $8.9 \pm 1.2\,\mu m$, for control and noise-exposed groups, respectively ($p > 0.05$). Similarly, neither, zero current potential (mean $Vz = -58.4 \pm 2.8\,mV$, control; $-62.8 \pm 3.3\,mV$ noise exposed), nor capacitance ($15.9 \pm 1.6\,pF$, control; $14.4 \pm 2.5\,pF$, noise exposed) varied between groups ($p > 0.05$). Application of ATP ($100\,\mu M$) to OHC under voltage clamp elicited rapid inward currents within a few hundred milliseconds, consistent with recruitment of P2XR conductance (Fig. 3a). Responses to ATP were recorded in OHC from both control (7/21 from 12 cochleas) and noise-treated cochleas (6/10 from 5 cochleas). The amplitude of the average initial ATP-gated current response was significantly greater in noise-treated OHC compared with cells from the control group ($-183 \pm 69\,pA$ and $-48 \pm 23\,pA$, respectively; $p < 0.05$). These ATP-gated currents were sustained for the duration of ATP application (1 s to 30 s), but exhibited run-down, or slow desensitisation, with repeated drug application. The P2X receptors were localised to the apical region of the rat OHC by focal application of ATP to specific sites (Fig. 3b), as demonstrated previously for guinea pig OHC [13] and mouse OHC [14]. The ATP-gated currents exhibited inward rectification of the I/V relationship (Fig. 3c).

DISCUSSION

The *in situ* hybridisation study indicated that $P2X_2R$ mRNA levels in the organ of Corti and SG were increased within a few hours of the onset of loud sound. In particular, the OHC and DC region exhibited considerable up-regulation, while intense punctate labelling was associated with individual primary auditory neurones in the ganglion. The immunohistochemistry indicated that sustained loud sound (3 days at 90 dB SPL) produced a significant increase in $P2X_2R$ protein expression in the regions shown to have $P2X_2R$ transcript up-regulation. Within the organ of Corti, the most apparent feature was the trafficking of additional $P2X_2R$ protein to the OHC cuticular plate region and stereocilia facing the endolymphatic compartment. This was functionally confirmed by obtaining electrophysiological recordings of ATP-gated currents in isolated OHC. The sustained ATP-gated inward currents recorded from the OHC in the present study are compatible with the phenotype of $P2X_2R$, and match those seen in guinea-pig and mouse OHC [4,14]. Noise exposure increased both the proportion of the OHC that responded to ATP and the mean amplitude of the ATP-activated currents.

The P2XR-mediated shunt conductance, which includes the hair cells and epithelial cells lining the scala media

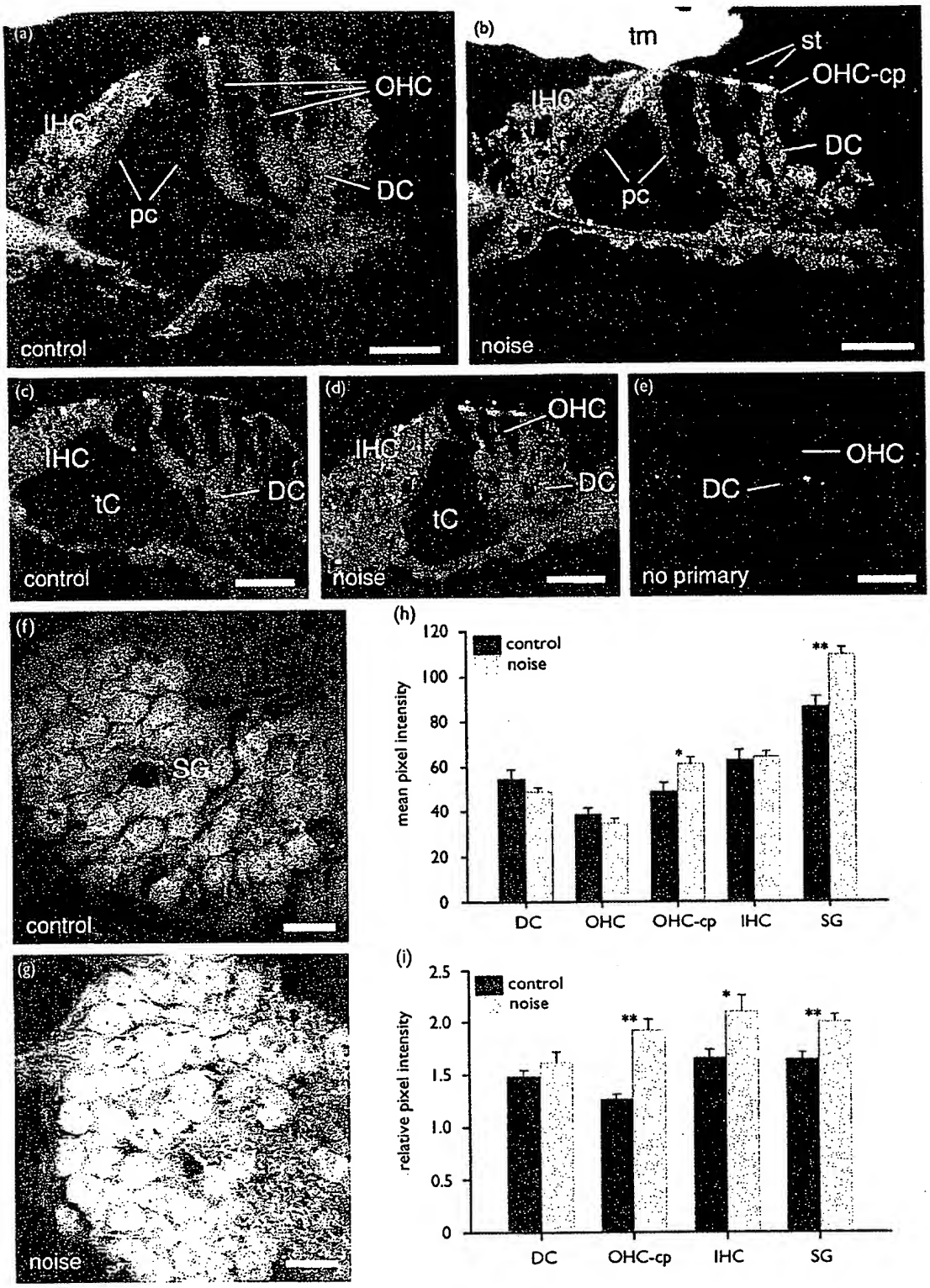

Fig. 2. Comparison of P2X$_2$R immunofluorescence labelling in the organ of Corti and spiral ganglion (SG) in control and noise-exposed tissue. (a) Confocal image of control organ of Corti (10 µm z stack reconstruction). Note the absence of label in the mid-region of the pillar cells (pc) forming the tunnel of Corti. (b) confocal image of noise-treated (90 dB SPL, 72 h) organ of Corti (10 µm z stack reconstruction). Note the pronounced immunofluorescence signal in the cuticular plate region of the outer hair cells (OHC-cp), and the outer hair cell stereocilia (st). (c–e) Confocal images of organ of Corti. (f) Spiral ganglion confocal image, control. (g) Confocal image of SG from noise-exposed cochlea. Note increase in labelling in neurones. (h) Comparison of mean pixel intensity in control and nose-exposed cochlear tissues. (i) Relative pixel intensity of cochlear tissues compared with the reference level in the sub-cuticular plate outer hair cell region (– see OHC, in h) and SG relative to the cochlear nerve (reference intensity level unchanged at ~55, not shown). Deiters' cells, DC; inner hair cells, IHC; tm, tectorial membrane; tC, tunnel of Corti. Bars = 25 µm.

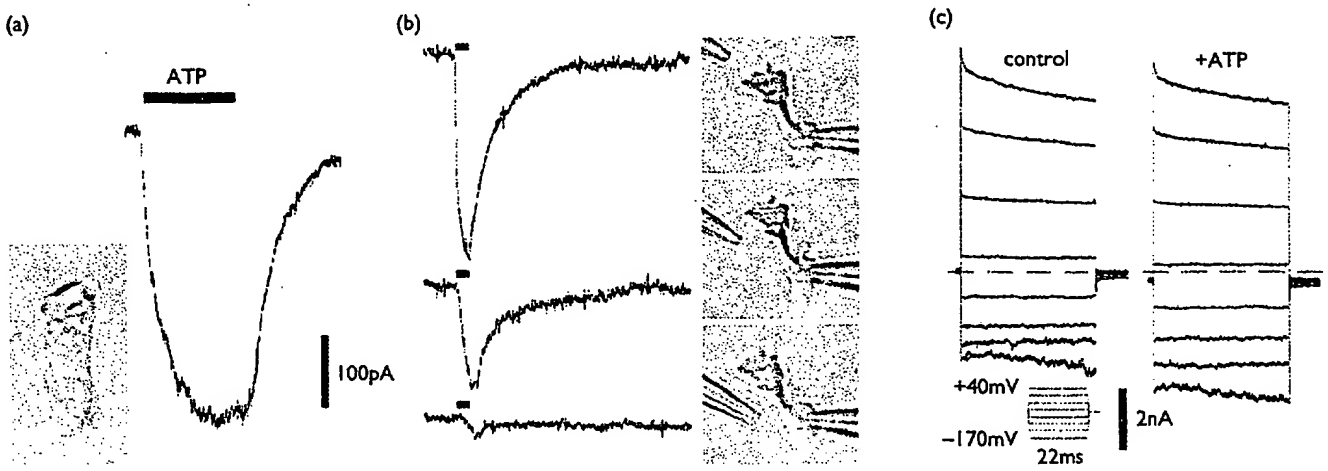

Fig. 3. Characterisation of ATP-gated current of rat outer hair cells (OHC). (a) Application of 100 μM ATP (bar = 5 s) via a drug pipette produced a rapidly activated, sustained inward current. OHC videomicrograph scale bar = 10 μm. (b) Localisation of ATP-gated inward currents to the apical (endolymphatic) surface of the cell (one-second applications; apex ATP-gated current amplitude = 262.4 pA, latency = 60 ms; middle = 133.8 pA, 270 ms; base = 41.3 pA, 1420 ms). (c) Inward rectification of ATP-gated current in an OHC demonstrated using a voltage-step protocol (250 ms duration, 30 mV steps; $V_h = -60$ mV). All records obtained from OHC isolated from noise-exposed cochlea. Holding potential −60 mV.

[9,12,15,21–23], provides a homeostatic regulation of the electrochemical gradient for sound transduction complementing autonomic action at the vascular stria [24]. Elevation of ATP within the scala media activates ATP-gated ion channels assembled from $P2X_2R$ subunits, either as homo-multimers, or hetero-multimers with other P2XR subunits, to reduce EP [12,23,24]. The noise-induced increase in $P2X_2R$ mRNA transcript and protein expression demonstrated here support enhanced ATP-gated shunt conductance. In OHC this appears to be particularly evident, where increases in $P2X_2R$ immunofluorescence ATP-activated conductance are associated with the site for sound transduction. Increased P2XR conductance at this site would reduce OHC transduction and hence the activity of the cochlear amplifier [13]. Whether this up-regulation of the $P2X_2R$ is triggered by sustained binding of ATP to the receptors, or is linked to other processes connected with the chronic sound stimulation, and hence depolarisation of the OHC, is unknown.

ATP can act as a co-transmitter or neuromodulator with other neurotransmitters, including glutamate [25]. While the effect of greater expression by the spiral ganglion neurones of ATP-gated ion channels assembled from $P2X_2R$ remains to be determined, it appears likely that ATP transmission influences the level of excitability of primary auditory neurones [26]. There also remains the possibility that the mix of P2XR subunits that determines the unusual phenotype of the spiral ganglion neurone ATP-gated inward current is altered by noise stimulation, as evidence to date indicates that the channels are hetero-multimers that include the $P2X_2$ receptor subunit [7].

CONCLUSION

Sustained loud noise produced an up-regulation of $P2X_2R$ expression in the rat cochlea. The increase in expression of these ATP-gated ion channel subunits was particularly evident at the site of outer hair cell sound transduction and may act to limit the focused amplification of sound energy, the physiological role of these cells. In addition, $P2X_2R$ expression also increased in spiral ganglion neurones, indicating that extracellular ATP features as a modulator of auditory neurotransmission that is adaptive and dependent on noise level.

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